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Antimycotic Screening of 58 Malaysian Plants against Plant Pathogens

Suhaila Mohamed,* Suzana Saka, Saleh H. El-Sharkawy, Abdul Manaf Ali & Sepiah Muid

Faculty of Food Science and Biotechnology, Universiti Pertanian Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Abstract: Ethanolic extracts of 58 Malaysian plants belonging to 24 different families were screened for antifungal activity against seven plant pathogens using the filter paper disc diffusion technique. Two varieties of *Piper betle*, showed strong activity against all the pathogens tested (*Colletotrichum capsici*, *Fusarium pallidoroseum*, *Botryodiplodia theobromae*, *Alternaria alternata*, *Penicillium citrinum*, *Phomopsis caricae-papayae* and *Aspergillus niger*), with inhibition diameters significantly ($P < 0{\cdot}01$) bigger than 2·5 mg ml^{-1} prochloraz or 10 mg ml^{-1} clotrimazole. The minimum inhibitory concentrations of the ethanolic extracts of *P. betle* against these plant pathogens ranged between 0·01 mg ml^{-1} and 1 mg ml^{-1}. Thirty-four other plants (Kucing gala, Limau batik, *Bertholletia excelsa*, *Bixa orellana*, *Caesalpinia pulcherrima*, *Cerbera odollam* (fruits and leaves), *Colocasia gigantea*, *Curcuma domestica*, *Curcuma manga*, *Derris eliptica*, *Elephantopus scaber*, *Eleusine indica*, *Eugenia polyantha*, *Euphorbia hirta*, *Euphorbia tirucalli*, *Gardenia florida*, *Hedyotis auricularia*, *Hibiscus rosa-sinensis*, *Juniperus chinensis* (*three varieties*), *Lawsonia inermis*, *Lecythis ollaria*, *Mentha arvensis*, *Mimusops elengi*, *Ocimum sanctum*, *Phyllanthus niruri*, *Piper nigrum*, *Piperomia pellucida*, *Pedilanthus tithymaloides*, *Polygonum minus*, *Spondias dulcis*, *Solanum nigrum*, *Tinospora tuberculata*) showed selective antifungal activity, while 21 species were inactive.

Key words: antifungal, *Piper betle*, plant pathogens, Malaysian plants

1 INTRODUCTION

Post-harvest infection on fruits limits the storage life and hinders the development of cultivation and marketing. The economic loss resulting from post-harvest fungal diseases may be 5–50%, or even higher in developing countries.[1] Fungi such as *Colletotrichum*, *Fusarium*, *Botrydiplodia*, *Alternaria*, *Botrytis*, *Monilia*, *Penicillium*, *Sclerotinia*, *Phomopsis*, *Aspergillus*, *Cladosporium*, *Corynespora* and *Rhizopus* spp. are known to cause serious fruit damage, depending on the type of fruit, clones, weather conditions and country of origin.[2,3]

Synthetic fungicides have been used for almost 20 years for the control of post-harvest diseases of tropical and subtropical fruits.[4] Many microbial pathogens, however, have begun to develop resistance to the most widely used chemicals, so there is a need to develop new fungicides with improved performance and less potential environmental impact. Relatively more plants have antibacterial than antifungal activity, which explains why plants are more resistant to bacterial attack than to fungal attacks.[5] This investigation attempted to identify new antifungal agents by screening 58 Malaysian plants against seven pathogen plant fungi.

2 MATERIAL AND METHODS

2.1 Plant material

Materials for screening were obtained from UPM botanical garden and identified by Dr Abd Ghani

* To whom correspondence should be addressed.

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Yunus, Director Plant Germplasm Department. Voucher herbarium specimens were prepared and deposited at the Department of Biotechnology, UPM. The tested plants were:

Acanthaceae: *Andrographis paniculata* Nees.
Anacardiaceae: *Spondias dulcis* Forst.
Apocynaceae: *Cerbera odollam* Gaertn., and *Plumeria rubra* L.
Araceae: *Colocasia gigantea* Hook.
Bixaceae: *Bixa orellana* L.
Compositae: *Cosmos cadatus* H.B. and K. and *Elephantopus scaber* L.
Coniferae: *Juniperus chinensis (three varieties)* L.
Euphorbiaceae: *Acalypha indica* L., *Euphorbia tirucalli* L., *Euphorbia hirta* L., *Excoecaria bicolor* Hassk., *Pedilanthus tithymaloides* Poit. and *Phyllanthus niruri* L.
Geraniaceae: *Averrhoa carambola* L.
Gramineae: *Cymbopogon citratus* (DC) Stapf., *Cymbopogan nardus* (L.) Rendle and *Eleusine indica* (L.) Gaertn.
Labiatae: *Mentha arvensis* L., *Ocimum sanctum* L. and *Orthosiphon stemineus* Benth.
Leguminosae: *Caesalpinia pulcherrima* L., *Cassia alata* L. and *Derris eliptica* Benth.
Liliaceae: *Allium schoenoprasum* L.
Lythraceae: *Lawsonia inermis* L.
Malvaceae/Bombacaceae: *Ceiba pentandra* (L.) Gaertn, and *Hibiscus rosa-sinensis* L.
Menispermaceae: *Tinospora tuberculata* Bumee.
Myrtaceae: *Eugenia michellii* Lam., *Eugenia polyantha* Wight, *Lecythis ollaria* L. and *Bertholletia excelsa* H.B. & K.
Piperaceae: *Piper nigrum* L., *Peperomia pellucida* H.B. and K. and *Piper betle* L. (two varieties)
Polygonaceae: *Polygonum minus* Huds.
Rubiaceae: *Hedyotis auricularia* L. and *Gardenia florida* L.
Sapotaceae: *Mimusops elengi* L.
Solanaceae: *Solanum nigrum* L., *Datura fastuosa* L. and *Capsicum annuum* L.
Umbelliferae: *Coriandrum sativum* L., *Cuminum cyminum* L., and *Centella asiatica* (L.) Urb.
Zingiberaceae: *Curcuma domestica* Loir., *Curcuma manga* Valeton, *Alpinia galanga* (L.) Willd., *Nicolaia speciosa* Horan and *Zingiber officinale* Roscoe.

Three plants of unknown scientific names were also screened, locally known as Asam katuk, Limau batik and Kucing gala.

2.2 Chemicals

Malt extract broth (MEB) and malt extract agar (MEA) were from Oxoid, Basingstoke, UK. Clotrimazole (UNIdrug house) and prochloraz (AgrEvo UK Ltd), were used as the standards for antifungal activity. Ethanol was obtained from BDH, UK.

2.3 Plant pathogens

Colletotrichum capsicii (H. Sydow) E. Butler & Bisby, *Fusarium pallidoroseum* (Cooke) Sacc., *Botrydiplodia theobromae* Pat., *Alternaria alternata* (Fr.) Keissler, *Penicillium citrinum* Thom, *Phomopsis caricae-papayae* Patrak and *Aspergillus niger* Van Tieghem were isolated directly from infected fruits and identified by Dr Sepiah Muid, a taxonomist in microbiology, at the Malaysian Agricultural Research and Development Institute, Serdang. Stock cultures were maintained on MEA slants at 4°C.

2.4 Plant extracts

Freshly collected leaves of green plants (unless otherwise specified) were sliced into small pieces and macerated with 80% ethanol with continuous shaking for 24 h at room temperature. The extract was filtered, and the filtrate was evaporated under reduced pressure at a temperature not exceeding 50°C. The dark brown viscous residue was reconstituted in ethanol (5 ml) and sterile filter paper discs (6 mm diameter) were impregnated with each plant extract solution. The discs were then left to dry at room temperature.

2.5 Antifungal activity[6]

The effect of the plant extracts on growth was studied using the disc diffusion method on MEA after incubation at 30°C for 72 h. One or two plugs of subcultured fungal colony were mixed homogeneously with 10 ml of sterile MEB. Innoculation was done by pipetting 0·1 ml of the suspended fungal spores on to sterile MEA in a petri dish and spreading it evenly with a hockey stick. The dried discs impregnated with plant extract were placed on the inoculated agar surface. Aqueous solutions of clotrimazole (10 mg ml^{-1}) and prochloraz (2·5 mg ml^{-1} were used as standards. The antimicrobial activity was recorded as the diameter (mm) of the clear inhibition zones surrounding the discs. Each test was repeated at least three times. The limitation of this method is that it will probably only show the activity of substances that can diffuse through or dissolve in the aqueous agar medium.

2.6 Minimum inhibition concentration[7,8]

Malt extract broth (0·5 ml) containing 10^7 fungal spores ml^{-1} was mixed with 1–2000 $\mu g\ ml^{-1}$ extracts from the two *P. betle* varieties, in ten-fold dilution assay, and incubated for 24–36 h at 30°C. Growth was measured by the optical density at 660 nm and the viability of the cultures was confirmed by incubation of the broth on malt extract agar plates. The concentration of the tube of the highest dilution that was free from growth was

recorded as the minimum inhibitory concentration (MIC, $\mu g\ ml^{-1}$).

3 RESULTS AND DISCUSSION

The fungi responded differently to the various plant extracts (Table 1). Thirty-five of the plants showed selective antimycotic activity, while the two varieties of *Piper betle* showed pronounced activity against all the pathogens tested, with inhibition zones significantly ($P < 0{\cdot}01$) bigger than those with $2{\cdot}5\ mg\ ml^{-1}$ prochloraz or $10\ mg\ ml^{-1}$ clotrimazole. The inhibition diameters (i.d.) for the two varieties of *P. betle* (red vein and green vein) were in most cases equivalent to or stronger than that for prochloraz, and about double that of clotrimazole. The estimated effective dose (minimum inhibitory concentration) of the ethanolic extracts of the two varieties of *P. betle* against the plant pathogens was very low, ranging from $10\ \mu g\ ml^{-1}$ to $1\ mg\ ml^{-1}$. *P. betle* was the only species that was active against all the pathogens tested. Considering that the yield of the extracts from the *P. betle* leaves is quite high ($(26 \pm 2)\ g\ 100\ g^{-1}$ dry weight) and the activity of the extracts is very strong, this plant is probably worth cultivating, for potential use of the antifungal compounds in in-vivo applications.

P. betle has antifungal, antiseptic and anthelmintic activity.[9] The high levels (1% of the fresh weight of the leaves) of five propenylphenols (chavicol, chavibetol, allylpyrocatechol, chavibetol acetate and allylpyrocatechol diacetate) were considered responsible for the fungicidal and nematocidal activity.[10] *P. betle* has been shown to be effective against plant pathogens such as *Pyricularia oryzae* Cav., *Cochliobolus miyabeanus* (Ito & Kurib) Drechsler ex. Dasture, *Rhizoctonia solani* Kuhn.,[11] *B. theobromae*[12] and *Thanatephorus cucumeris* (Frank) Donk[13] which are responsible for collar rot. This work confirmed the activity against *B. theobromae* and showed it to be strongly active against six other plant pathogens, namely *Colletotrichum capsici*, *Fusarium pallidoroseum*, *Alternaria alternata*, *Penicillium citrinum*, *Phomopsis caricae-papayae* and *Aspergillus niger*.

Of the taxa surveyed *H. rosa-sinensis* and *C. odollam* also showed significant antimycotic activity against *B. theobromae* with i.d. of 19 mm and 17 mm respectively. The activities of these two plants were about equal to $10\ mg\ ml^{-1}$ clotrimazole and about half as effective as $2{\cdot}5\ mg\ ml^{-1}$ prochloraz. *B. theobromae* causes stem end rots or rots to fruits that are bruised and can be controlled by treatments such as double hot water dips with thiazole.

J. chinensis (var. 3) and *E. polyantha* showed inhibition towards *A. alternata* with an i.d. of 15 mm, which is about half of that with clotrimazole and one-third that with prochloraz. *Alternaria* cause spotting in stored fruits. *E. indica*, *E. hirta*, *M. elengi*, *C. odollam* (fruits), *B. orellana* and *C. pulcherrima* extracts were less effective against *A. alternata* (i.d. 10–13 mm). Plants with weak activities (i.d. 7–9 mm) against *A. alternata* were *J. chinensis*, *E. tirucalli*, *H. auriculata*, *C. gigantea*, *P. nigrum*, *P. minus*, *P. niruri*, *P. pellucida* and *S. dulcis*.

H. auricularia and *L. inermis* extracts were effective against *P. citrinum*, with i.d. of 15–16 mm, which is about two-thirds that with the clotrimazole standard and half that with the prochloraz standard. *Penicillium* spp. cause about 30% post-harvest losses, especially in pomegranates and citrus fruits.[14] Other plants that displayed some activity (i.d. 10–13 mm) against *P. citrinum* were *J. chinensis* (var. 3), *C. manga*, *S. nigrum*, *N. arvensis*, *G. florida* and *P. pellucida*. Plants that showed weak activities (i.d. 7–9 mm) against *P. citrinum* were *J. chinensis* (var. 2), *D. eliptica*, *T. tuberculata*, *C. domestica*, *E. indica*, *E. hirta*, *P. niruri*, *B. excelsia* and *S. dulcis*.

J. chinensis (var. 2) and *C. manga* showed inhibition towards *P. caricae-papayae*, with i.d. of 15 mm which is about two-thirds that of the standards. *Phomopsis* infection occurs less extensively than anthracnose on bruised fruits, causing colour loss and soft, enlarged, waterlogged flesh, but treatments with propiconazole or prochloraz are less effective against *Phomopsis* than against anthracnose. Other plants that showed some activity (i.d. 10–13 mm) against *Phomopsis* included *E. indica* and *L. inermis*, while *E. hirta*, and *B. excelsa* showed weak activities (i.d. 8–9 mm). *J. chinensis* (var. 3), *B. excelsa*, *L. inermis*, *S. dulcis* and *C. pulcherrima* extracts were active against *Colletotrichum capsicii* (i.d. 10–15 mm i.e. about half of the standards). *Colletotrichum spp.* cause anthracnose in various fruits whose onset can only be delayed or reduced but not eliminated by preharvest and post-harvest treatments. Other plants that showed weak activities against *C. capsicii* were *E. indica*, *E. hirta*, *C. odollam* (both fruit and leaves), *B. orellana*, *P. nigrum*, *E. polyantha*, *P. tithymaloides*, *G. florida*, *P. niruri*, *L. ollaria* and *P. pellucida*.

Other than *P. betle*, *M. arvensis* and *J. chinensis* (var. 3) were the only extracts showing good activity against *F. pallidorosum*, with inhibition diameters of 12–13 mm, i.e. half of the standards. *Fusarium* spp. attack only a small percentage of fruits under prolonged storage at low temperature, and infection can be effectively controlled by post-harvest treatments. Plants that showed weak activities (i.d. 7–9 mm) against *F. pallidorosum* were *J. chinensis*, *E. indica*, *E. hirta*, Limau batik, *C. mangga*, Kucing gala, *P. niruri*, *P. pellucida*, *E. scaber*, *B. excelsia* and *S. dulcis*.

J. chinensis (var. 3), *L. inermis*, Kucing gala and *P. niruri* showed some activity towards *A. niger* with i.d. of 9–13 mm i.e. half the activity of $10\ mg\ ml^{-1}$ clotrimazole and about one-third that of $2{\cdot}5\ mg\ ml^{-1}$ prochloraz. *Aspergillus* can be controlled by careful post-harvest handling and treatment, but become

TABLE 1
Antimycotic Activity of the Tested Plant Extracts on Plant Pathogens (Positive Results Only) after 72 h Incubation

Plant	Diameter of inhibition (mm)[a]						
	Aa	*Bt*	*Cc*	*Pc*	*Ps*	*Fp*	*As*
Bertholletia excelsa	—	—	14	8	9	9	—
Bixa orellana	13	—	7	—	—	—	—
Caesalpinia pulcherrima	10	—	12	—	—	—	—
Cerbera odollan (fruit)	10	—	8	—	—	—	—
Cerbera odollan (leaves)	—	19	8	—	—	—	—
Colocasia gigantea	7	—	—	—	—	—	—
Curcuma domestica	—	—	—	7	—	—	—
Curcuma manga	—	—	—	11	15	7	—
Derris eliptica	—	—	—	6	—	—	—
Elephantopus scaber	—	—	—	—	—	7	—
Eleusine indica	10	—	9	8	10	8	—
Eugenia polyantha	15	—	9	—	—	—	—
Euphorbia hirta	8	—	7	9	8	8	—
Euphorbia tirucalli	8	—	—	—	—	—	—
Gardenia florida	—	—	9	10	—	—	—
Hedyotis auricularia	7	—	—	16	—	—	—
Hibiscus rosa-sinensis	—	17	—	—	—	—	—
Juniperus chinensis (variety 1)	—	—	—	7	—	—	—
Juniperus chinensis (variety 2)	7	—	—	7	15	8	—
Juniperus chinensis (variety 3)	15	—	11	11	—	12	11
Kucing gala	—	—	—	—	—	8	11
Lawsonia inermis	—	—	10	15	13	9	13
Lecythis ollaria	13	—	7	—	—	—	—
Limau batik	—	—	—	—	—	7	—
Mentha arvensis	—	—	—	10	—	13	—
Mimusops elengi	10	—	—	—	—	—	—
Ocimum sanctum	8	—	—	—	—	—	—
Pedilanthus tithymaloides	—	—	9	—	—	—	—
Phyllanthus niruri	8	—	8	9	—	8	9
Piper betle (green vein)	42	33	34	40	35	36	34
Piper betle (red vein)	49	35	33	37	35	34	38
Piper nigrum	7	—	8	—	—	—	—
Piperomia pellucida	8	—	8	13	—	9	—
Polygonum minus	8	—	—	—	—	—	—
Solanum nigrum	—	—	—	13	—	—	—
Spondias dulcis	9	—	10	9	—	8	—
Tinospora tuberculata	—	—	—	9	—	—	—
Standards							
Clotrimazole (10 mg ml^{-1})	26	17	22	22	21	21	20
Prochloraz (2·5 mg ml^{-1})	50	33	28	32	26	28	35
Minimum inhibitory concentrations of *Piper betle* ethanol extracts ($\mu g\ ml^{-1}$)							
Piper betle (red vein)	1000	100	1000	100	1000	1000	100
Piper betle (green vein)	10	10	100	10	1000	1000	1000

[a] Plant pathogens:
Aa: *Alternaria alternata* Bt: *Botrydiplodia theobromae*
Cc: *Colletotrichum capsicii* Pc: *Penicillium citrinum*
Ps: *Phomopsis caricae-papayae* Fp: *Fusarium pallidoroseum*
As: *Aspergillus niger*.

serious in bruised fruits or those stored for a long time at 10–15°C.

P. niruri, which is active against *C. capsicii*, *F. pallidoroseum*, *A. alternata*, *P. citrinum* and *A. niger*, is known as a liver-protecting plant in traditional medicine.[15] The activity is due to its anti-hepatotoxic lignins, nirphylin (lignan) and phyllnirurin (neolignan), which were identified as 3,3,5,9,9,-pentamethoxy-4-hydroxy-4,5-methyl-

dioxylignan and 3,4-methylenedioxy-5-methoxy-9-hydroxy-4,7-epoxy-8,3-neolignan.[16]

L. inermis plant extracts were effective against *C. capsicii*, *P. citrinum*, *P. caricae-papayae* and *A. niger*. The active compound in *L. inermis* could be lawsone.[17]

J. chinensis extracts were effective against *C. capsicii*, *F. pallidoroseum*, *A. alternata*, *P. citrinum* and *A. niger*.

E. hirta has been found to contain terpene ester derivatives which have cocarcinogenic activities, and can be used against leukemia.[18–20] *C. manga*, which tastes like unripe mango, contains 1–3% volatile oil, mainly terpenes, camphor and turmeron.[21,22] *P. nigrum* contains piperine, pellitorine and other unsaturated isobutylamines[23] shown to be active against *Rhizoctonia azolla* pathogen[24] and fruitfly.[25] *C. domestica* produces volatile oils, containing tumeron and dehydrotumeron, which have anti-*E. coli* activity,[26] and repel insects.[22]

Of the plants studied, the two varieties of *P. betle* showed significant antifungal activity against all the pathogens tested compared to the standards. Further investigations will be carried out to study *in-vivo* applications. There are several varieties of cultivated *P. betle*: 1. Sireh cengkeh (clove betle) has small leaves and a distinct clove-like flavour; 2. Sireh Melayu (Malay betle) has green veins; 3. Sireh Cina (Chinese betle) has large leaves and delicate flavour; 4. Sireh udang/India (Shrimp/Indian betle) has red vein and petiole; 5. Sireh hitam (black betle) has small firm, strong-tasting dark leaves; and 6. Sireh buah (fruiting betle) has coarse leaves and fruits readily. Factors that affect the flavour, hence the chemical constituents of the betle leaves include the variety, age of the plant, degree of exposure to light and position of the leaf on the vine. Chewing of *P. betle* leaves acts as a gentle stimulant, sweetens the breath, and acts as medicament from diseases of the mucous membranes of nose and the alimentary canal. In Malay medicine, the juice from *P. betle* leaves is used as eyedrops for eye injury/infection, as a baby lotion for the newborn, for coughs, asthma, constipation and to arrest milk secretion.[27] Eugenol has been identified as the major constituent of the volatile compounds in *P. betle* and the active component against *Pisum sativum*.[28]

The plants that showed no antimycotic activity against the plant pathogens tested were *A. paniculata*, *P. rubra*, *C. cadatus*, *A. indica*, *E. bicolor*, *A. carambola*, *C. citratus*, *C. nardus*, *O. stemineus*, *C. alata*, *A. schoenoprasum*, *C. pentandra*, *E. michellii*, *P. minus*, *D. fastuosa*, *C. annuum*, *C. sativum*, *C. cyminum*, *H. asiatica*, *A. galanga*, *P. speciosa*, *Z. officinale*.

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